CORYNEBACTERIUM DIPHTHERIAE

A Correlation of Recorded Variations within the Species[1]

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Diphtheria is one of the diseases either diagnosed or confirmed in the laboratory by the isolation and identification of the causative microörganism, therefore it is of great practical as well as theoretical importance that we know as much as possible concerning the range of variation and the dissociative behavior of the causative organism commonly referred to as "the diphtheria bacillus." The organism has been studied extensively, but often the investigations were confined to certain aspects while totally ignoring other aspects or even previous studies along the same lines. It is not surprising then to find in the literature numerous conflicting reports on the behavior of the diphtheria bacillus.

Our present pleomorphic concept of bacterial species is one which depicts order and not chaos within each species. For several bacterial species it has become possible to correlate many of the chemical and physical properties of the microörganisms with colony form. A preliminary review of the literature (Morton, 1935b) suggested that this is also possible in the case of the diphtherial species. It is from this point of view that the experimental work reported elsewhere (Morton, 1940) was carried forward and the following review prepared.

So-called dissociative studies of recent times have contributed to a better understanding of a bacterial species; and the variations already reported fall into a generally consistent pattern. In the case of the diphtheria bacillus, a consideration of the earlier

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reports of its variation shows that, on the whole, the isolated observations do not represent incongruities within the species but rather an orderly trend in the variation process. Many of the seemingly independent variations can now be correlated with variations in colony form. So evident is this that it is often possible to predict many of the properties of a culture by merely establishing the colony form to which it belongs. Despite the relatively short period in which the various culture phases have been recognized as such, there is present in the older literature much valuable information which, if rightly interpreted, contributes to our better understanding of the variation problem.

The review which follows endeavors to treat only of the nature and degree of the recorded variations of the diphtheria bacillus and the extent to which they may be correlated with the recognized colony forms. The characteristics or properties are considered in the following order: (1) Colony forms; (2) Appearance of growth in liquid media; (3) Stability of the cell suspensions in salt solution; (4) Virulence; (5) Toxigenicity; (6) Serological reactions; (7) Hemolysis; (8) Fermentation reactions; (9) Chromogenesis; (10) Reduction of potassium tellurite; (11) Cell morphology.

1. COLONY FORMS

The smooth (S), intermediate (SR), and rough (R) colony forms

Six years after Loeffler's original description of the colonies produced by the diphtheria bacillus, variations were reported by Klein (1890). Peters (1897) also noticed a variation in the colonies, but the first to give serious attention to colony variation of the diphtheria bacillus were Cobbett and Phillips (1897). In addition to describing "shiny" and "matt" colonies, Zupnik (1897) confirmed the observations of Cobbett and Phillips that the composition of the medium is very important in determining the colony form. Serum or glycerol agar do not differentiate the shiny and matt colonies, but the difference can be observed on ordinary agar. These findings were confirmed by Schick and Ersettig (1903); and numerous others have reported the occurrence of two distinct colony forms for the diphtheria bacillus.

Following Arkwright's (1921) coinage of the terms *smooth* and *rough* to describe the forms of the colonies produced by the intestinal group of microörganisms, and the application of these terms to the colony forms of other species, Scott (1923) named the two kinds of colonies of the diphtheria bacillus as smooth and rough. Cowan (1927) has claimed a separation of the Park No. 8 and of another strain into smooth and rough colonies by selective cultivation; however, one wonders if she had the pure S form, because her smooth colonies are described as "less raised and less dense" than the R colonies. The purity of her R form is also open to question, for it is stated to produce a smaller pellicle than the S. A very important observation by Miss Cowan is that in the development of the R form the organisms are seen to pass through a stage in which the colonies are, for the most part, very large, irregular and coarsely granular, and the individual bacilli are also larger and of a great variety of shapes, often showing branched forms. Parker (1928) mentions smooth and rough strains, but here again the findings are not in accord with what one usually thinks of as the actual smooth and rough colony forms. Okell (1930) was not able to confirm Cowan's work even with the same cultures used by the latter; but this is not at all unusual since cultures very often cannot be dissociated at will. Yü (1930) was able to dissociate the S colony form to what he regarded as the R but was unable to change the R back to the S. Viewing the pictures of his colonies, however, one wonders if he had the actual R form.

Study of the colony form of the diphtheria bacillus assumed a different aspect following the description by Anderson, Happold, McLeod, and Thomson (1931) of "gravis" and "mitis" forms. After routine laboratory examinations over a period of eight years, they concluded that there are two distinct, stable types of the diphtheria bacillus with which are associated the following characteristics.

Type *gravis*. The growth on potassium tellurite chocolate medium after 48 hours is gray or gray-black. The colony is like that of *C. xerosis*, but heavier. The cell morphology on media, other than Loeffler's, is that of a very short diphtheroid, usually without granules. On

Loeffler's medium the granules are sometimes well marked and at other times scanty. There is a pellicle and granular deposit in broth; and the organisms give rise to unstable suspensions in saline, are non-hemolytic, possess typical fermentation reactions (no acid with sucrose, acid with glucose, maltose, galactose and invariably with dextrin, starch and glycogen), and grow vigorously on the special chocolate tellurite medium. The organisms are associated with severe cases of diphtheria.

Type *mitis*. The growth on the potassium tellurite chocolate medium after 48 hours is black. The colony is like that of *C. hofmanni*, but finer and more translucent. The cell morphology is that of the text-book *C. diphtheriae*, the bacilli usually being long and the granules well marked. There is uniform turbidity in broth; and the organisms produce stable suspensions in saline, are hemolytic, give recognized fermentation reactions, (no acid with starch or glycogen and inconsistent results with dextrin), and are partially inhibited upon subculturing to the chocolate tellurite medium. The organisms are associated with mild cases of diphtheria. The type *mitis* is commonly non-virulent for guinea pigs, but the type *gravis* almost always shows some degree of virulence.

Later (1933), Anderson and his colleagues proposed a third, type *intermedius*, that was said to be associated with diphtherias of intermediate severity. Its description follows.

Type *intermedius*. The type of growth on potassium tellurite chocolate agar, serum agar, heated blood agar, or plain agar is fine. The colonies are flat with central knob and slightly crenated periphery. On potassium tellurite chocolate agar the colonies are very fine with slightly raised black center and thinner translucent periphery. There is granular growth in broth, which settles rapidly. Neither starch nor glycogen is fermented. The morphology is that of a barred diphtheroid with metachromatic granules variable but often poorly developed.

There are at least two serious faults in the above descriptions and assumptions. First, not much information is conveyed in a description which states that a colony form is like that of *C. xerosis* or of *C. hofmanni*, as bacteriologists have realized since the works of Arkwright (1921) and Hadley (1927, 1937) demonstrated that no microörganism has a single and distinctive colony

form. The color plates, however, convey a fairly good picture of the two colony forms, which Anderson, *et al.*, were attempting to describe. Second, it is highly questionable whether or not it is possible to associate slight variations in clinical manifestations with colony form. Menton (1932) and others believe that the terms "gravis" and "mitis" have no biological basis. Although Anderson, Cooper, Happold, and McLeod (1933), and Carter (1933) claim that the colony forms and fermentation reactions are stable over a long period of cultivation, such claims are not in strict agreement with our present conceptions of bacterial variation. It is not surprising then that other workers are in disagreement, notably Wright and Rankin (1932), and Menton, Cooper, Duke, and Fussell (1933). Evidence of variation of the types *mitis* and *gravis* is given by Christison (1933) who observed a type *mitis* strain change its manner of growth in broth from turbidity and compact sediment to that of a pellicle and granular sediment with the supernatant fluid remaining clear, by subculturing in broth of pH 7.8 at three-day intervals. In addition, Menton was able to transform the type *gravis* colony to the type *mitis* form by the addition of commercial antitoxin to the medium. The work of Robinson (1934) shows that the various type strains of *C. diphtheriae* are relatively stable in ordinary stock cultures, but that by the usual *in vitro* and *in vivo* methods employed to promote variation, these strains show marked variation in most of the type characteristics. These latter findings are in keeping with our concepts of the dissociative behavior of a bacterial species. Menton (1932), Murray (1935a), and others have observed that typical colonies of one form may have the biochemical characteristics of another colony form; and numerous workers, in addition to Robinson and Peeney (1936), have contributed evidence for the variations in type characteristics of the strains.

Not only is type *gravis* not always associated with severe cases of diphtheria, nor type *mitis* with mild cases, but occasionally both types are isolated from patients or found in stock cultures. Parish, Whatley and O'Brien (1932) found that the mortality rate in human beings is about the same for organisms of both

colony forms and that, over a large region, the type *gravis* strains have been reported to be as prevalent in mild cases as in severe cases. Although the range in virulence of type *mitis* strains for guinea pigs was found by Anderson, *et al.*, to be greater than that of type *gravis* strains, the latter showing more uniform virulence, only three of the 90 strains tested for virulence by these workers, (employing the intracutaneous method on guinea pigs), gave a very marked reaction and these were type *mitis* strains. Whatever the type, 50 per cent or more, of the strains produced a moderate reaction in the guinea pigs. Bearing in mind the work of Powell (1923), who found that 40,000 to 400,000 diphtheria bacilli are required for a minimal reacting dose in the intradermal method of estimating virulence, one may well hesitate to interpret a reaction to the test dose as doubtful, slight, moderate, marked or very marked, unless the dose is more sharply standardized. If administered either subcutaneously or intradermally into guinea pigs, the type *mitis* strains are as virulent as the type *gravis* strains (Parish, Whatley, and O'Brien). When administered either intravenously or subcutaneously into rabbits, the type *mitis* strains appear to be more virulent.

It is quite obvious from the data that no one particular colony form exemplifies the diphtherial species. Diphtheria bacilli are either virulent or nonvirulent, and, if virulent, this quality is not necessarily associated with any particular colony form as, for example, is the case with the pneumococcus. The quantitative differences between virulent strains of diphtheria bacilli can be explained on the basis of S-R variation.

Later and more detailed reports of rough colonies of the diphtheria bacillus are those by Whitley (1934), who found a "rough" form present in nose and throat cultures; by Morton (1935a, 1940) and Hobby (1935), who produced R forms by forced dissociation; and by Bisset (1938) who encountered R colonies in strains which were being repeatedly subcultured. Thus far, of all the descriptions of the rough colony forms, those by Morton and by Hobby appear to represent the ultimate rough form, in that they more nearly fulfill the criteria for a rough culture phase as described by Hadley. Following are the criteria for a rough,

or R, colony form, in general. The margin is very irregular; the surface is very flat, uneven and granular; the organisms grow as a granular sediment in broth and clump spontaneously in physiological salt solution. Evidence for this type of colony does not appear very often in the literature, in spite of the fact that workers have designated some of their colony forms as rough.

The usual criteria for the smooth, or S, colony form, in general, are: the margin is round and even; the surface is convex and smooth; the organisms grow in broth with a uniform turbidity, and produce a stable suspension in physiological salt solution. Workers prior to Baerthlein and Arkwright did not always search for these cardinal points, but from their meager descriptions one is able to recognize some of the colonies, undoubtedly, as of the smooth form. The first impression one receives from the description by Anderson, Happold, McLeod, and Thomson (1931) is that their type *mitis* colonies are the smooth form. This impression is verified when one examines the colonies by some special means of illumination, as did Murray (1935b).

During the transformation of the smooth to the rough colony form, the surface of the colonies becomes flattened, irregular and granular; and, as Cowan mentioned, the colonies also become larger. It is this intermediate form, SR (somewhat removed from the pure S), which produces the pellicle type of growth with sediment and clear supernatant, so often obtained with diphtherial cultures. One immediately recognizes the similarity of the type *gravis* colonies described by the English workers and the SR colony form.

From the description by Anderson, *et al.*, it appears that some of their intermediate forms, which could not be classified as either type *mitis* or type *gravis* colonies, represent, perhaps, a stage of the diphtherial organism somewhat removed from the SR stage and approaching roughness, whereas other forms in this group are suggestive of a dwarfed smooth colony. The term "intermediate", when used in this connection does not necessarily mean that such a colony is intermediate between those of types *mitis* and *gravis*. In the terminology employed in respect to smooth and rough colonies, the term "intermediate" signifies

that the colony has an appearance that is in between those of the smooth and the rough forms; the "intermediate" colony can be observed to arise from one form and proceed into the other.

When one reads the reports of the incidence of types *gravis* and *mitis*, and intermediate forms in various localities and epidemics, it is readily apparent that the predominating type is likely to vary from one locality to another and from one epidemic to another. If virulence is not necessarily associated with one particular colony form of the diphtherial species, as numerous workers have shown, then it is possible for one colony form to predominate in a given epidemic or in a certain locality and to be replaced by another colony form during another epidemic or in another locality, which, in fact, is substantiated by the findings of Frobisher (1939).

In no other species of microörganisms have colony forms been named according to the quantitative variations in clinical manifestations which they produce, and, from the number of discordant reports, this does not appear to be the most satisfactory basis for nomenclature for the diphtherial species. The inadequacy of the classification is exemplified by the necessity for the creation of sub-groups, as was done by Wright, Christison, Rankin, Pearson, and Cuthbert (1935), and Stuart (1938), who found no correlation between virulence, colony form, and the ability of a strain to ferment starch. It is surprising that earlier students of types *gravis* and *mitis* did not attempt to associate the various colony forms with S-R variation, a system of variation and nomenclature which has been found applicable and useful for hundreds of other bacterial species.

Dwarf colony form (D)

It is interesting that in the same year in which colonial variation of the diphtheria bacillus first received serious study, dwarf colony forms were encountered. It was only the larger forms, however, the smooth and rough colonies, which were studied in the years that followed. Cobbett and Phillips in their studies in 1897 described extremely minute colonies of the diphtheria bacillus. When 10 cultures of virulent diphtheria bacilli were

grown on gelatin, 6 of them yielded two varieties of colonies, designated as large and small colony types. Two cultures yielded only the small colony type and two the large type only. When separated into pure cultures, the large and small colony forms maintained for many generations their characteristic difference in size. From pure cultures of the large colonies, all the colonies in subsequent generations were of the same large type. Pure cultures of the small colonies, however, although continuing to produce small colonies for many generations, occasionally gave rise to colonies of the large type. One strain maintained constantly the property of producing only the small colony form. Cobbett and Phillips at first were inclined to ascribe this phenomenon to their failure to separate completely the two varieties and not to a tendency to variation; but later they ruled out the possibility of impure cultures. The appearance of the two colony forms on gelatin was the only instance in which the strains varied, the strains were alike in virulence, microscopic appearance, and in their manner of growth in broth and upon media other than gelatin. These small colonies were extremely minute, often scarcely visible, and seldom as large as a half-millimeter in diameter.

According to the type of growth on trypsin-serum agar, Parker (1928) temporarily classified diphtherial cultures into heavy and light growers. The heavy growers were further classified into R-heavy and S-heavy growers. The light growers were not as easily differentiated from the heavy growers on serum agar in which the serum had not been trypsinized nor on plain agar. The light growers were in every case (16 strains) toxigenic. These light growers described by Parker, appear similar to the small colony forms described by Cobbett and Phillips, and may well be the forms which at the present time are referred to as dwarf (D) forms.

The "intermediate" type (of Anderson *et al.*) when plated from broth cultures was observed by Christison (1933) to yield frequently two kinds of colonies. One kind was irregular, granular, and flat with a central knob, which is suggestive of an sR colony form, and the other was much darker, glistening and

entire, but smaller and more convex than type *mitis* colonies of the same age. Since Christison made no statement as to the exact size of the colonies, it is difficult to interpret this colony form, but there is a possibility that it might be the dwarf colony form of the diphtheria bacillus.

Bacilli from type *gravis* colonies have also been observed to give off small-colony variants. Erzin (1936) describes two forms of growth for this type: typical type *gravis* colonies and "zarte" colonies. On blood agar the "zarte" colonies are round, convex, small, and hemolytic. On Clauberg's medium the colonies are soft, delicate, and tiny. On the medium of Gundel and Teitz the colonies are very small, delicate, appearing scarcely visible through a magnifying glass, and somtimes convex and shiny. On Loeffler's medium and in broth the organisms produce "typical growth for diphtheria." Upon the same five media, the type *gravis* or "typical" colonies produce "typical growth." Incubation of a broth culture of the "zarte" form at 37°C. gives rise after 10 days to a mixture of "zarte" and type *gravis* colonies. Incubation of a broth culture of type *gravis* at 37°C. gives rise after 34 days to a mixture of the two forms. The "zarte" form produces toxin but is less virulent for guinea pigs than type *gravis*.

Morton (1935a) reports small colony variants of the diphtheria bacillus, which are designated dwarf or "D" colonies. The small colony variants were produced by the aging of broth cultures of a Park No. 8 strain. The organisms are pathogenic for guinea pigs, are toxigenic, and ferment glucose and dextrin but not sucrose. They are agglutinated by immune serum prepared by immunizing rabbits against the parent culture of the Park No. 8 strain. In two filtration experiments, the organisms were not found to pass through a Berkefeld N filter. Hobby, in 1935, reported that her R type colony from a recently isolated diphtherial strain (RB-2T) either remained as such or was transformed to a minute or "G" colony.

Two forms of small colonies were observed by Mittag (1937) in addition to the commoner large forms. The small, irregular colonies, ("kleine, zarte, gekerbte Kolonien") appear to be the sR form, and the small, delicate, round colonies ("kleine, zarte,

runde Kolonien") appear to be the dwarf colonies. The organisms were pathogenic for guinea pigs.

Our review shows that small colony forms have been described for the diphtheria bacillus on at least six different occasions. The ability to produce an extremely small colony form, in addition to the more common large colony forms, is not a unique characteristic of the diphtherial species but seems to be a property inherent in many bacterial species. Similar forms have been described for the following: *Bacillus megatherium* (Rettger and Gillespie, 1933), *B. mesentericus* and *B. vulgatus* (Flynn and Rettger, 1934), *Clostridium welchii* (Roe, 1932, 1934), *Diplococcus pneumoniae* (Dawson, 1928; Eaton, 1934), *Eberthella typhosa* (Fromme, 1911; Eisenberg, 1914), *Hemophilus pertussis* (Kimball, unpublished data), *Lactobacillus acidophilus* (Kopeloff, 1934), *Neisseria gonorrheae* (Raven, 1934), *Pseudomonas fluorescens* and *Ps. pyocyanea* (Eisenberg, 1914), *Salmonella aertrycke* and *S. schottmuelleri* (Fürth, 1922; Koser, 1930), *S. suipestifer* (Orcutt, 1923), *Shigella equirulis* (Edwards, 1931), *S. dysenteriae* Shiga (Arkwright, 1921), *S. sonne* (Koser and Styron, 1930; Koser and Dienst, 1934; Chinn, 1936), *Staphylococcus aureus* (Swingle, 1934) and a group C hemolytic streptococcus (Morton and Sommer, unpublished data). In many of these cases the small colony forms were called dwarfs, and in other cases they were called "G" colonies in spite of the fact that they did not satisfy the criteria for the G type colony. If more care had been exercised in the classification of small colony forms, less confusion would now exist concerning the G forms. These dwarf forms are not at all unusual. One notices that they may occur under normal conditions of cultivation, but that their ratio to the larger colony forms is quite small. Frequently the dwarf colony forms are produced under the influence of aging or by growth in the presence of lithium chloride. One also recognizes that they are produced from cultures that are showing some evidence of, or a tendency toward, the rough phase. When practically the same phenomenon has been observed and described for at least 20 bacterial species, representing more than 12 different genera, it would seem to warrant recognition as a definite phase in bacterial variation.

Gonidial colony form (G)

In addition to the small colony forms mentioned above, which have been described by various workers since 1897, a few of the observations warrant special attention, because a small colony (G) form was obtained under special conditions of cultivation, and because it represents growth from *filterable* elements of the bacteria. In this respect the G colony form is unique. Hauduroy (1927) obtains such colonies from filtrates of diphtherial cultures by serially culturing the filtrates on solid media. This is accomplished by seeding a few drops of the filtrate on an agar plate. After suitable incubation the surface of the plate is washed with a few drops of sterile broth, and the washings are transferred to another fresh, sterile plate, this process being carried forward in series. Smith and Jordan (1930) describe growths from 11 of 19 filtrates of aged diphtherial cultures after serial transfers of the filtrates as recommended by Hauduroy. Hadley and Richardson (Hadley, Delves and Klimek, 1931) and Morton (1932) also report small colonies (designated as G colonies) from filtrates of diphtherial cultures.

Since many workers have been erroneously calling all small colonies G colonies through failure to establish that the organisms therefrom fulfill the necessary requirements, these criteria as proposed by Hadley (1931) will be reiterated. They are as follows:

"1. Visible signs of culture development appear in the filtrate, or in subcultures from it, only after a considerable delay, perhaps after from 6 to 12 days, or even after a longer period in the case of some species.

"2. Colonies on agar plates are very slow in appearing, perhaps after from 24 hours to 8 days, and the first colonies to arise are of minute size, having an average diameter of 0.2 mm. or less at the end of 96 hours on a favorable medium present in sufficient depth in the plate.

"3. The morphologic type of the organisms present in the filtrate, or in subcultures or colonies, is composed mainly of minute coccus forms, granular bodies and delicate skeins or filaments, with a suggestion of diplococcus or streptococcus arrangement (Giemsa staining); also occasional coccobacilli.

"4. In organisms the 'normal' form of which is acid-fast or gram-positive, the staining reaction of the recovered culture elements is temporarily reversed or variable.

"5. The biochemical and serologic reactions of the recovered cultures are different from those of the parent culture, and virulence, toxicity, and susceptibility to the homologous bacteriophage are altered."

Of course, contamination is ruled out by eventually regaining the original culture form after it has gone through its filterable stage.

Mucoid colony form (M)

Moist colonies have been described for the diphtheria bacillus, but Hobby (1935) was the first to name a mucoid phase of the diphtheria bacillus. It has not yet been demonstrated, however, that diphtheria bacilli in the mucoid phase possess a capsule nor that they possess more of the type-specific substance than when in any other colony phase.

Summary. Thus, during the period of more than half a century that has elapsed since the original observations of the colonies produced by the diphtheria bacillus, many colony forms have been noted. These various colony forms were either described, or may now be interpreted, as the mucoid (M), smooth (S), intermediate (SR), rough (R), dwarf (D) and gonidial (G). Photographs and additional detailed descriptions of these different culture phases will be found in the paper by Morton (1940). The terms "mitis," "gravis," and "intermedius" as applied to the colony forms of the diphtheria bacillus do not truly designate the clinical manifestations which were originally thought to be associated with these cultural phases. Since the form of colony to which these terms originally referred can be described adequately by the older and accepted terminology (smooth and rough colony forms), there appears to be no need for the continuance of the terms "mitis," "gravis" and "intermedius" in that respect.

2. APPEARANCE OF THE GROWTH OF SMOOTH, INTERMEDIATE, ROUGH AND DWARF COLONY FORMS IN LIQUID MEDIA

The manner of growth of a strain in broth often conveys as much information relative to the culture phase as does the appear-

ance of the colonies on solid media. In addition to confirming the observations of Roux and Yersin (1888) that diphtheria bacilli grow in broth as fine granules which adhere to the walls of the vessels, Brieger and Fraenkel (1890) observed that in some cases the bacilli grow to produce a homogeneous and uniform turbidity. The first indication of a correlation of such types of growth with differences in colony form in the diphtherial species came from the work of Baerthlein (1913) and of Bernhardt (1915) who reported that cells from a large, moist colony form grew with an even turbidity, whereas those from a fine, translucent, bluish, and brittle colony form grew only as a heavy, granular precipitate. The large, moist colony form described by Baerthlein produced a black color when grown on tellurite medium; and the fine, translucent, bluish form of colony produced only a light brown color. This represents the first indication that appearances on tellurite medium, in addition to the manner of growth in broth, may also be associated with colony form. Riemsdijk (1914) described three types of growth in broth, all of which can now be correlated with colony form: (1) granular growth on the bottom of the tube, associated with the rough colony form; (2) homogeneous clouding, associated with the smooth colony form, and type *mitis* colony described by the English workers; and (3) occasional pellicle formation, associated with the intermediate (SR) colony form and type *gravis* colony. The mucoid form is described by Hobby as growing with a uniform turbidity, similar to that produced by the S form. Neither the M, S, nor R forms grow in a granular pellicle (Hobby). These isolated observations have been confirmed by Morton (1940), who found in addition that the D form grows with a very faint turbidity, which is often finely granular. The G form often fails to impart visible signs of growth in filtrates for a considerable time. When visible signs of growth do occur, it is as a very fine turbidity with a ropy sediment. The statement in Bergey's manual that the diphtheria bacillus grows in broth as a "fine, granular deposit on sides and bottom of tube, forming a thin, fragile pellicle on neutral medium" is inadequate.

3. STABILITY OF THE CELL SUSPENSIONS IN SALT SOLUTION

The spontaneous clumping of diphtheria bacilli in physiological salt solution has often been very disconcerting. Indeed, Priestley (1911) reported that an agglutination test for the identification of *C. diphtheriae* was impractical since 12 out of 15 strains tested by him clumped spontaneously. The addition of glycerol, formalin, various salts, etc., failed to prevent the spontaneous clumping. Early, unsuccessful attempts were made to associate the property of remaining in uniform suspension in physiological salt solution with virulence or toxigenicity. Scott (1923) reported that such spontaneous clumping is not consistently associated with either the presence or absence of toxigenicity; therefore, it is not surprising that Okell (1930) and Jones (1930) were unable to find any significant relationship between agglutinability in salt solution and virulence. Although Scott found no complete association between roughness of growth and sensitiveness or insensitiveness to electrolytes, more recent studies have shown that sensitiveness to electrolytes is associated with colony form. Yü (1930) and Morton (1940) both found that the smooth colony form yields a uniform suspension in physiological salt solution. (The "mitis" form, which may be interpreted as a smooth colony form, gives uniform suspensions). The rough colony form was found to give granular and unstable suspensions. In a comparative study of this property among the S, SR, R, and D colony forms of diphtheria bacilli (Morton, 1940), it was found that the S and D forms give uniform suspensions in salt solutions in a concentration of 0.2 to 8 per cent, organisms in the R form clump spontaneously, and those intermediate between the S and R give indeterminate results.

4. VIRULENCE

It seems to be agreed that diphtheria bacilli, as identified by colony form, fermentation reactions, agglutination tests, etc., may be either virulent or non-virulent for guinea pigs, *i.e.*, the microörganisms, when injected into guinea pigs, may or may not bring about the death of the animals. The strains, whether

virulent or non-virulent, undergo the same variations in colony form. Earlier workers believed that diphtheria bacilli vary in virulence, ranging from non-virulent to highly virulent. Recent workers employing single-cell cultures and the intradermal method for estimating virulence find that diphtherial cultures are either virulent or non-virulent, none being intermediate (Powell, 1923; Dudley, 1925 and Okell and Parish, 1926). The work of Powell, in addition to showing that the virulence of diphtheria bacilli for guinea pigs varies only within narrow limits, showed that 40,000 to 400,000 organisms are required for a minimal reacting dose by the intradermal method, whereas 14 million to 140 million, or 35 times as many organisms are required when employing the subcutaneous method for estimating virulence. This, while establishing the superiority of the intradermal over the subcutaneous method, still leaves a rather wide latitude in the effective number of microörganisms.

Powell found that single-cell strains have the same degree of virulence as their respective parent cultures and different single-cell strains derived from the same single-cell strain are equally virulent for guinea pigs. However, since Roux and Yersin (1890) first reported that virulent diphtheria bacilli may give off non-virluent variants, other observers have noted the same phenomenon. In view of the fact that other species of virulent microörganisms give off non-virulent variants, the same process may be readily accepted for the diphtheria bacillus. Reports of the reverse process, that of non-virulent variants reverting to virulent forms, are far less common, and some believe that it cannot take place. Trumpp (1896) claimed to have transformed an avirulent strain into one possessing virulence by injecting the culture into guinea pigs along with sublethal doses of toxin. Morton (1940) reported that after two cultures of the virulent Park No. 8 strain had aged in ampoules 132 and 140 days, respectively, the organisms produced S colonies on solid medium whereas the strain originally was of the SR colony type. Upon five occasions, cultures from the S colonies were non-virulent for guinea pigs. After 14 generations on plain or blood infusion agar, one of the S strains killed guinea pigs in doses of 37,000,000 or-

ganisms administered intraperitoneally. An explanation for this reversion might be that during the physiological youth of the culture, it had not attained its maximum toxin-producing properties, just as was shown (Morton, 1940) that during the physiological youth of a diphtherial culture great variations in morphology may take place.

A study of colony forms has not contributed anything toward an explanation for virulent and non-virulent forms of the diphtheria bacillus. Cobbett and Phillips (1897) noticed no difference in virulence between the strains derived from their various colony forms. The dissociative studies by Hobby were made, unfortunately, on a strain which did not possess virulence. In the studies by Morton on virulent strains, the various colony forms (S, SR, R, and D) retained their pathogenicity for guinea pigs. Yü (1931) reported that his S, virulent form, isolated during the acute stages of diphtheria, was followed during convalescence by non-virulent S and R forms. Nevertheless, virulence is not associated with colony form as Yü thought it to be. This is evident from the studies by Cobbett and Phillips and by Morton, and is borne out by the reports cited in the next section on toxigenicity, where there are listed the results obtained from the use of culture filtrates instead of whole cultures.

It is rather instructive to examine the most recent description of the pathological effects of diphtheria bacilli in man as observed by McLeod, Orr, and Woodcock (1939) on the morbid anatomy in gravis, intermedius, and mitis diphtherias from a series of 51 necropsies. Their study indicates that an infection with type *mitis* (S form) diphtheria bacilli is of less serious menace to the individual than an infection with organisms of type *gravis* (SR form). In acute cases the primary lesions caused by types *gravis* and *intermedius* are differentiated from those of type *mitis* (S form) on the following points: much less superficial membrane in the fauces and less tendency to extend into the intrathoracic air passages; the membrane less firm in texture; deeper penetration of the tissues of the inflamed parts and greater involvement of the cervical lymph glands and surrounding tissues; and selective involvement of the tonsils and the deeper tissues at this site.

The most frequent causes of death were (a) respiratory obstruction as the result of membrane formation, which was more frequent with type *mitis*; and (b) toxic effects on the viscera, especially the heart and kidneys, which were more frequent with types *gravis* and *intermedius*. Thus, although virulence is not associated with colony form, the above evidence suggests that there may be a correlation between the colony form and certain pathologic characteristics.

Since the type *gravis* usually ferments glycogen in the test tube and type *mitis* does not, Knox and Passmore (1938) reasoned that the fermentation of glycogen might be the explanation for the greater virulence of type *gravis* within the human body. However, their finding by metabolic studies that both forms utilize glycogen eliminated this possible explanation. Povitzky, Eisner, and Jackson (1933) produced death in guinea pigs, weighing about 300 gm., in 24 to 48 hours with a Park No. 8, a type *mitis* and a type *gravis* cultures, employing the growth from $1\frac{1}{3}$, $\frac{9}{10}$, and $\frac{1}{3}$ veal-agar slants, respectively. Their explanation for the fact that the type *gravis* culture was more virulent than the others was that it probably produces toxin more readily in the animal body. Their *in vitro* results, however, do not bear out this reasoning, for in 48 to 72 hours, cultures from the above three strains contained 550, 350 and 200 M.L.D. of toxin per ml., respectively. Their *in vivo* results, in view of the findings of McLeod, Orr, and Woodcock, may be explained from another angle.

The vital question of why certain strains of diphtheria bacilli are virulent for man, guinea pig, or other animals, while other strains are not, is still unanswered. The property of virulence is not associated with colony form nor is toxigenicity the complete explanation. Perhaps careful chemical or metabolic studies will bring forth the long awaited answer.

5. TOXIGENICITY

In contrast to the previous section, wherein results of infection of the animal body with the various colony forms of the diphtheria bacillus were discussed, this section is concerned primarily with

the ability of these organisms to elaborate a toxin, as judged by testing the cell-free culture filtrate. Many of the technical details concerned with the production of diphtherial toxin have been described by Andrewes, *et al.* (1923). Investigators agree that diphtheria bacilli, as identified by colony forms, fermentation reactions, agglutination tests, etc., may or may not elaborate the specific exotoxin. Crowell (1926), working with a single-cell strain, observed that it is possible for a toxigenic strain to give off both toxigenic and non-toxigenic descendants. He, like others, never observed a non-toxigenic strain to regain its toxigenicity.

Toxigenicity is not necessarily associated quantitatively with virulence. It is generally known that the Park No. 8 strain, which produces one of the most potent diphtherial toxins, is not one of the most virulent strains. The work of Povitzky, Eisner, and Jackson (1933) showed quantitatively that the virulence and toxigenicity of different strains may vary independently of one another.

Toxigenicity is not associated with any serological type. Havens' (1920) report that the toxins elaborated by two serological types were not identical, created considerable consternation, for bacteriologists and clinicians had taken it for granted that there is but one diphtherial toxin. This report by Havens, however, was disproved by Paxson and Redowitz (1922), Hartley (1923), and Park, Williams, and Mann (1922). The latter workers found that the toxins elaborated by organisms of the various serological types are qualitatively, and from the practical standpoint, quantitatively alike.

It has been demonstrated that toxigenicity is not associated with colony form. The smooth and matt colony forms of Schick and Ersettig (1903) produced toxins which were neutralized by diphtherial antitoxin. Although Cowan (1927) and Yü (1931) claimed that their R strains failed to produce toxin, Povitzky, Eisner, and Jackson (1933) found that both types *mitis* and *gravis* produce toxin; and more recently Morton (1940) reported that the S, SR, R, and D colony forms produce toxin.

No matter in what serological group or colony form a diph-

theria bacillus may be, the toxin, if it is produced, is one and the same. There are, however, differences in the rate of production and the amount produced. The reason why certain strains of diphtheria bacilli are able to elaborate the exotoxin and other strains are not, is still unanswered. Dissociative studies and investigations into the antigenic structure of the bacterial cell have thus far failed to reveal the answer. Perhaps a study of the enzyme systems of the diphtherial cell will furnish an explanation.

6. SEROLOGICAL REACTIONS

Early investigators soon learned that there is no relationship between the agglutinability and the virulence or toxigenicity of diphtheria bacilli. Moreover, the agglutination reaction has failed thus far to reveal any changes in cellular antigens of virulent diphtheria bacilli when the bacilli become non-virulent. Also, it has not been possible to discover a diphtherial agglutinating serum capable of agglutinating a pseudo-diphtheria bacillus or a diphtheroid. Nor does a diphtherial agglutinating serum agglutinate all strains of diphtheria bacilli. The more this species is subjected to serological study, the greater is the number of serological types that are discovered. Lipstein in 1903 recognized four definite serological types of *C. diphtheriae*; and Durand (1918, 1920) reported five and a group of heterologous strains. Havens (1920) temporarily complicated matters by reporting only two serological groups but these findings were soon disproved by Bell (1922) and by Park, Williams, and Mann (1922). The latter found at least five serological types. Smith (1923) reported seven serological types; and Robinson and Peeney (1936), in a study of 739 strains, encountered a fifth type in addition to Ewing's (1933) four. Eagleton and Baxter (1923) found ten types, as did Sia and Huang (1939). Murray (1935c) reported eleven types and a group of unclassifiable strains. Undoubtedly more will be reported in the future.

Practically the same results have been obtained by agglutinin-absorption as by the agglutination test. The agglutinin-absorption procedure enables more strains to be classified because

spontaneous agglutination of the strains does not interfere with the text.

Only recently has the question of serological types been approached from a different angle of investigation, namely, by the attempt to isolate the chemical substance responsible for type-specificity. Wong and T'ung (1938) reported at first that the polysaccharides of *C. diphtheriae* are shared by types *mitis*, *gravis*, *intermedius*, and by an avirulent strain, and later (1939) that the polysaccharides are group-specific. It is quite possible that Wong and T'ung selected cultural variants of the same serological type for these studies. After Sia and Huang (1939) found different cultural forms within the same serological type, Wong and T'ung in their later work (1939, 1940) selected strains from different serological types, instead of from different cultural forms, for their chemical studies. As a result, they were able to isolate chemically a substance which reacts only with serum prepared against the homologous strain. To date the preliminary findings of Wong and T'ung are that the cellular constituent of the diphtherial cell which is responsible for serological type-specificity is an alkali-soluble protein that is heat-labile, and convertible to a group-specific protein by heating at 56°C. for 30 minutes.

In 1903 Schick and Ersettig reported that organisms from their smooth and matt colonies are agglutinated by the same antiserum; and additional studies along this line by Morton (1940) showed that organisms in the S, SR, R, and D colony forms react similarly against type-specific serum. Single-cell strains from different colony cultures have the same agglutinative reaction as the parent strain (Powell, 1923).

The attempt on the part of the English workers to identify their types *mitis* and *gravis* by fermentation reactions and antigenic types appears to be impractical. It has been shown that microörganisms of one colony form may have the fermentation reactions of those of the other colony form. This, moreover, has been verified by studies in metabolism which reveal that microörganisms of both colony forms possess similar enzyme systems. Furthermore, it is not in keeping with our general

knowledge of bacterial species for different serological types within the species to be characterized by distinctive colony forms. In their investigations on the chemical substances within the diphtherial cell, Wong and T'ung found that a type-specific substance is present in strains of different cultural reactions, but it is present only in strains of the same serological type.

Inasmuch as the organisms in at least one of the major colony forms of the diphtherial species clump spontaneously in physiological salt solution and a fair percentage of diphtherial cultures encountered in nature give unstable suspensions, agglutinin-absorption or chemical fractionation of the organisms and precipitin tests, of necessity, have to be employed for serological typing. The chemical studies begun only recently by the Chinese workers should prove to be of great value. The early report that the bacilli from smooth and matt colonies are agglutinated by the same serum, and the preliminary finding that bacilli in the S, SR, R, or D colony forms behave similarly towards type-specific sera indicates that type specificity is not associated with a particular colony form, as is the case with *Diplococcus pneumoniae*.

7. HEMOLYSIS

It has been known since the report of Eijkman (1901) that diphtherial cultures are able to hemolyze red blood corpuscles, but that the hemolytic power is variable. The hemolytic substance is present in living cultures only, is destroyed by heat, and is non-filterable (Schwoner, 1904; Wesselow, 1914; Costa, Troisier, and Dauvergne, 1918). Practically all investigators have found that the hemolytic property in corynebacteria is associated only with the diphtherial species; strains of *C. xerosis* and pseudo-diphtheria bacilli lack this property. Red blood cells from different species of animals vary in susceptibility in the following decreasing order: rabbit, guinea pig, goat, dog, horse, and human (Schwoner, 1904; Goldie, 1933).

There is no correlation between the hemolytic power and the toxigenicity of a strain (Maunu, 1914; Heeren and Megrail, 1930), nor virulence nor the agglutination reaction (Hammer-

schmidt, 1924). Anderson, Happold, McLeod, and Thomson (1931) reported their type *mitis*, which we now know to be the smooth colony form, as hemolytic and their type *gravis*, which may be interpreted as an SR colony form, as non-hemolytic. The work of Christison (1933) brought out more strikingly the correlation of hemolytic power with colony form. She found that the rough strains derived from type *mitis* (smooth) colonies either fail to hemolyze red blood cells or give only faint traces of hemolysis. Smooth strains derived from type *gravis* colonies lyse red blood cells more frequently than do the rough strains.

In a comparative study of the hemolytic activity of the various colony forms, Morton (1940) reported that organisms in the S colony form are hemolytic, less so in the R, and the dwarf forms are non-hemolytic.

8. FERMENTATION REACTIONS

Andrewes, *et al.* (1923) in their monograph on diphtheria give a historical survey of the development of fermentation tests for diphtheria bacilli and the diphtheroids. All workers agree that *C. diphtheriae* ferments glucose with the production of acid only, and that dextrin is usually fermented. Sucrose is usually not attacked; but Martin (1898), Cary (1917), Durand (1921), Fitzgerald and Doyle (1923), and Wright and Rankin (1932) have reported instances in which diphtherial strains have fermented sucrose. However, in view of the rarity of sucrose-fermenting strains of diphtheria bacilli, the readiness with which sucrose solutions may become altered during sterilization by heat or simply through the aging of aqueous solutions, and the diversity of media and pH indicators employed in fermentation tests, it might be well for this property to be reinvestigated by careful metabolic studies.

Schick and Ersettig (1903) reported that organisms from both their smooth and matt colonies fermented glucose; and more recently Cowan (1927) and Yü (1930) reported that their smooth and rough colony forms had the same fermentation reactions, the only difference being that the R form required a longer time to bring about the reaction. Judging from the descriptions or

photographs submitted by Cowan and Yü, it is not certain that they were working with the true S and R forms. At any rate, organisms in the S, SR, R, and D colony forms have the same fermentation reactions (Morton, 1940). Organisms from SR colonies are the most rapid fermenters, whereas the D forms require the longest period of time to bring about the production of acid. The differences between the various colony forms are quantitative rather than qualitative, a condition which is usually the case with the various colony forms within a species.

Anderson, *et al.*, (1931) observed qualitative differences in the fermentation reactions for different colony forms of the diphtherial organism. They reported inconsistent results with type *mitis*, while type *gravis* invariably fermented dextrin. Sharper differences, however, were encountered with starch and glycogen, which were fermented only by type *gravis*. The more vigorous fermentative activity of organisms in the SR colony forms (Morton) may explain the greater frequency with which type *gravis* attacked dextrin. Not always does type *mitis* (S form) fail to attack starch and glycogen, for Carter (1933) reported 1.17 per cent of the cultures from 510 positive swabs as being aberrant in that starch and glycogen were fermented but that the organisms grew in broth with a turbidity and no pellicle, a condition which is very suggestive of the S form. Robinson and Peeney (1936) also observed strains which were smooth and fermented starch, and strains which in other cultural respects resembled type *gravis* but failed to ferment starch. Ewing (1933) found two of 106 strains that fermented starch and produced type *mitis* colonies. Moreover, Wright and Rankin (1932) reported that none of their strains isolated from 50 cases of diphtheria fermented starch. They also found that one type *mitis* and two intermediate strains in addition to the type *gravis* strains fermented glycogen.

From the foregoing, one must conclude that the different colony forms of the diphtherial organism can not be differentiated definitely by qualitative differences in the usual fermentation tests. The clinching evidence for this decision would be metabolic studies with suspensions of the bacterial cells, and so far as the author is aware the only metabolic studies planned to clarify

this point have been those of Passmore (1938) and Knox and Passmore (1938). The latter workers found that there is no significant difference between the oxygen consumptions per hour per mg. dry weight of bacterial suspensions of types *mitis* and *gravis* when employing glucose or glycogen as the substrate. On the other hand, when tested in the usual way with glycogen in fermentation tubes, type *gravis* produces a strongly acid reaction, while type *mitis* remains neutral and, in addition, shows no utilization of the glycogen. According to Knox and Passmore, the discrepancies in results obtained when using fermentation tubes are accounted for by the differences in aeration within the tubes.

The property of utilization of carbohydrates by the diphtheria bacillus needs to be reinvestigated by more accurate metabolic studies. The great variations in manner of growth in liquid medium unquestionably account for great differences in aeration within fermentation tubes. The studies by Passmore show that enzymes for breaking down carbohydrates, when present in only small amounts, may be completely suppressed in organisms during their growth on nutritive media. At present there is no evidence substantiating the claim of Anderson, *et al.*, that the colony forms can be differentiated by qualitatively different fermentation reactions. The diphtherial organisms behave like other bacteria in that the various colony forms have the same fermentation reactions, though there are, perhaps, quantitative differences.

9. CHROMOGENESIS

Numerous workers have observed the production of a pigment in diphtherial cultures. Andrewes *et al.* (1923) state that this property is probably more dependent upon the composition of the medium than upon the organism, but at the present time it is not known what is responsible for the pigmentation which is observed occasionally. A yellowish color was observed by Zupnik (1897), by Cobbett and Phillips (1897), and by Morton (1940). Baerthlein (1913) and Hadley (1927) observed the yellowish color among R variants while Baerthlein (1918) and

Ewing (1933) observed it among S strains. It is thus evident that pigmentation is not associated with any particular colony type, the pigmentation being observed in the S and R forms with about equal frequency. A citron-yellow color was reported by Przewoski (1912) and by Bernhardt and Paneth (1913) to be quite common. Heinemann (1917) found diphtherial cultures taking on a yellowish-brown color, as did Bernhardt (1915) and Hill (1903); the latter also reported a pink color.

No investigations were made upon the pigments until Smith (1930) observed in filtrates of diphtherial cultures a pigment which he identified as a porphyrin. He was not able to establish any relationship between the rate of formation of the pigment and the rate of toxin formation. Although Coulter and Stone (1931) reported a direct relationship between the biological titer of culture filtrates and porphyrin content, the work of Wadsworth, Crowe, and Smith (1935) suggests that the substances are not one and the same, because they observed that the "selective absorbing ingredient" could be removed from culture filtrates with activated charcoal without significantly lowering the toxicity of the filtrate. Also the results with an ultrafiltered toxin showed that the ingredient, which is soluble in ether, was present in both the toxic residue and the non-toxic filtrate. This pigment showed absorption bands similar to those of some of the porphyrins and appeared to be produced or liberated by the diphtheria bacillus under conditions also favorable for toxin production. These studies were carried out with toxigenic and non-toxigenic strains; the various colony phases of the organism were not touched upon. In a somewhat broader study, Wheeler (1940) found porphyrins present in cultures of 10 non-virulent, non-toxigenic strains of *C. diphtheriae*, of 3 strains of *C. ulcerans*, 2 strains of *C. ovis* and 1 strain of *C. hoagii* but not in cultures of 3 strains of *C. xerosis* and 1 strain of *C. hofmanni*. These observations are in accord with those of Wadsworth, Crowe, and Smith, in that the conditions under which pigment and toxin production are observed in cultures of virulent, toxigenic diphtheria bacilli are similar to those under which non-virulent bacilli and related species of *Corynebacterium* synthesize porphyrins.

10. REDUCTION OF POTASSIUM TELLURITE

The cells of *C. diphtheriae* become colored by the reduced metal when the culture is grown in the presence of salts of tellurous acid (Klett, 1900). Conradi and Troch (1912) proposed a medium containing tellurite for the isolation of diphtheria bacilli, because on this medium the colonies assumed a characteristic black color. Since then numerous authors have proposed methods and media based on this phenomenon for the rapid isolation of diphtheria bacilli from mixed cultures. Baerthlein's (1913) observation that a large, moist colony type produced a black color when grown on Conradi's medium, which Conradi considered typical for *C. diphtheriae*, and a fine, translucent, bluish type of colony produced only a light brown color is the first suggestion of the variation in the color of diphtherial colonies on tellurite media being associated with colony form. It is upon the appearance of the diphtherial colonies on potassium tellurite-chocolate agar that Anderson and his co-workers based their classification and judged the pathogenicity of the microörganisms for human beings. We have already seen that virulence and toxigenicity of the cultures are not associated with colony form. On the other hand, pigmentation of the colonies on the special tellurite medium is a property which can apparently be correlated with the colony form.

In connection with the reduction of potassium tellurite by diphtheria bacilli, Manzullo in 1938 described a rapid diagnostic *in vivo* test for diphtheria. His method is to touch the exudate or pseudomembrane in the back of the throat with a swab which has been dipped in a 2 per cent solution of potassium tellurite. In the case of diphtheria, the area thus treated is supposed to turn black in 5 to 10 minutes. In the original series of 75 patients, Manzullo obtained no false positive results and only 7.4 per cent of the cases of diphtheria gave negative reactions to the immediate tellurite test. The experiences of other investigators with this test have differed from that of Manzullo very strikingly. Fox, Rhoads, and Lock (1939) applied the test to 27 patients with throat infections. All of them gave a negative tellurite test in spite of the fact that *C. diphtheriae* was isolated on Loeffler's

medium from 17 of the patients. There was one false positive reaction among 10 cases of non-diphtheritic infections. Tomlin (1939) found 22.7 per cent false positives. Tombleson and Campbell (1939), in a study of 200 patients, reported agreement between the tellurite test and the bacteriological diagnosis in 67.5 per cent, and with the clinical diagnosis in 77 per cent of the cases. Definite blackening occurred in specimens from 84.3 per cent of the cases which were finally diagnosed as diphtheria. Blackening also occurred in 46.8 per cent of faucial lesions caused by organisms other than the diphtheria bacillus. In a study of 277 swabs from 84 patients, Cooper, Peters, and Wiseman (1939) experienced 22.8 per cent false negative reactions in 57 bacteriologically proven cases of diphtheria, and 55 per cent false positives in 27 patients in which *C. diphtheriae* was never demonstrated. Four strains of *C. diphtheriae* were isolated from Loeffler's medium which failed to appear on the potassium tellurite plates. Tynan (1939) reported 13 per cent false negative and 47 per cent false positive reactions in his study of 75 unselected patients. Murray (1939) in a study of 62 individuals found that 36.6 per cent of the non-diphtheritic infections in addition to 84.3 per cent of the diphtheria patients gave a positive Manzullo test. Of those individuals giving a negative test 20 per cent were definite cases of diphtheria. Among the series of 200 cases studied by Woodcock (1939), 113 diphtheria patients gave a positive swab in 91 per cent, and a positive Manzullo test in only 85 per cent of the cases. Of 39 non-diphtheria patients, 95 per cent gave a negative swab whereas only 21 per cent gave a negative test; of 48 doubtful diphtheritic infections, 73 per cent gave positive swabs and only 48 per cent gave a positive Manzullo test.

The consensus among those who have investigated the Manzullo test is that it is unreliable and cannot replace the bacteriological methods in use at the present time. It is not unreasonable to expect that, if colonies composed of diphtheria bacilli show a variation in the reduction of potassium tellurite, the pseudomembranes in the throat, also composed of diphtheria bacilli, will show a variation in the reduction of the tellurite salt. In

addition, one cannot ignore the fact that blackening of tellurite is a reduction process that may under suitable conditions be carried out by agents other than the diphtheria bacillus.

Anderson, Happold, McLeod, and Thomson reported that organisms from type *mitis* colonies were partially inhibited upon subculturing to chocolate tellurite agar. Morton (1935b) observed the inhibition of growth on chocolate tellurite agar of the S colony form as well as type *mitis*; and more recently Cooper, Peters, and Wiseman (1939), and Perry and Petran (1939) have cited instances where diphtherial organisms were isolated from Loeffler's medium but not detected on tellurite medium. If it is true that type *mitis* represents the smooth phase and type *gravis* some phase of the culture nearer the rough phase, then, on the basis of the properties associated with smooth and rough colony phases as cited by Hadley in 1927, one would expect type *gravis* to be more resistant to inhibitory substances than type *mitis*.

11. CELL MORPHOLOGY

Many factors, both intrinsic and extrinsic, exert an influence upon the morphology of the bacterial cell. It was recognized early that there are many morphological forms of the true diphtheria bacillus; and Wesbrook, Wilson, and McDaniel (1900) attempted to classify the various forms, a classification which occasionally is used even today. They knew, of course, that cultures of *C. diphtheriae* are composed, not of one, but of many morphological forms. Only those forms which predominated, or were present in fairly large numbers were recorded for the culture. Almost all of the forms listed in their classification could be observed in any one culture if sufficient time was spent in the examination. Since the advent of the single-cell technic, the conclusion prevails that the various morphological forms of the diphtheria bacillus are of no hereditary significance, as the subcultures of a single cell of any morphological form may show a mixture of many morphological forms. This fact was demonstrated by Powell (1923) and Crowell (1926) and has been confirmed by all who have cultured single cells of this organism.

The various morphological forms that have been reported for the diphtherial organism may be listed as follows:

(*A*) *Rod forms.* By far the most common morphological form of the diphtherial organism is the rod. This form, however, is subject to a great many variations. Some of the factors which bring about such variations will be mentioned shortly. As a working classification, and based upon the appearances of the microörganisms after being stained with Loeffler's methylene blue, Wesbrook, Wilson, and McDaniel grouped the morphological forms into granular rods, barred rods and solid-staining rods. Granular-barred forms were reported later (Schultz, 1909). These various rod forms may be grouped into four major groups, viz.: (a) granular rods, (b) granular-barred rods, (c) barred rods, and (d) solid-staining rods.

The significance of these forms is still a disputed problem, although the subject has received much attention because of its practical relation to the matter of diagnosis. These forms are readily changeable from one to another as numerous workers have shown. Wherry (1917) advanced the hypothesis that there is a normal evolution beginning with the D^2 type or smaller and developing successively into the D^1, C^1, A^1 and sometimes the A type (Wesbrook classification). Others have agreed with this suggestion.

(*B*) *Thread and branching forms.* Long thread forms showing true branching were observed early in smears from diphtheritic membranes and in cultures. Egg media, serum agar, and potato media appear to favor the production of branching forms although they are observed in cultures on ordinary agar. Grasset and Grasset (1930) have described thread forms that were produced by growing the organism in the presence of bile. Thread forms from cultures that were suggestive of approaching roughness were seen by Neisser (1932) and similar forms, definitely associated with the rough colony forms, were described by Hobby (1935).

(*C*) *Spheroidal forms.* Under certain conditions it appears that the morphology of the diphtherial organism may assume a spheroidal form (Grubb and Koser, 1934). The coccus-like

cells of *C. diphtheriae* seem to be of two types: (1) those produced temporarily by the environment and not stable; and (2) those which seem to be stabilized and to be the result of some intrinsic change within the organism, such as the C-forms of Kuhn and Sternberg (1931) and the coccoid form reported by Stone and Hobby (1934).

The cell morphology of the diphtheria bacillus is of great practical significance because many tentative diagnoses are based upon it. One must be familiar with the influences which the colony phase and the environment may have upon the shape of the bacilli. Many of the factors are listed below.

Relation to colony form. When it was apparent that certain cultural characteristics often could be associated with colony form, it was to be expected that in the diphtherial species, as in many other bacterial species, certain morphological forms would show correlation with colony form. In the case of mucoid (M) colonies, no encapsulated organisms have been demonstrated. According to Hobby (1935), the organisms are longer and more granular than those obtained from smooth colonies. Organisms from smooth (S) colonies show more uniform rod forms than those from SR colonies. Although the cells are rod-shaped and stain unevenly, their outlines are not distorted to the same extent as are those from cells in the SR colony form (Morton, 1940). In the intermediate (SR) colonies, the cells are larger and the shapes more bizarre than is the case for cells from the other colony types (Cowan, 1927; Morton, 1935b). Organisms from the rough (R) colonies are long and filamentous, often forming long, intertwining threads (Hobby, 1935). Grasset and Grasset (1930) did not mention the colony phase in their report on the filamentous forms; but one recognizes that the cultures described by Neisser (1932) as filamentous forms ("Fadendiphtheriebazillen") were approaching the rough phase.

There is little information relative to the morphology of the organisms in the small colony forms prior to the studies described by Morton, (1935b), who found that organisms in the dwarf (D) colonies are short and somewhat thick with some tendency towards swollen forms and uneven staining by methylene blue.

Usually the cells stain solidly with the gram and methylene blue stains. They exhibit the typical arrangements of cells so common among corynebacteria. Organisms in the G colonies are very short, slender rods, sometimes nearly spherical. They are variable towards Gram's stain and stain unevenly with methylene blue.

Different sections of the same colony were observed by Zarniko (1889) to show variations in morphology and size of the diphtheria bacilli. This may be due to the differences in age of the various bacilli within a given colony or it may be due to the differences in the immediate surroundings of the bacilli in different sections of the same colony; both factors, as we shall see, are capable of exerting an influence upon cell morphology.

Relation to age of culture. Even during the normal growth of diphtherial organisms under the most favorable conditions, there is a great variation in the morphology of the cells. Many changes in morphology occur during the first 15 hours of growth on Loeffler's medium (Denny, 1903). The young cultures show solid-staining forms, and as the cultures grow older these forms give rise to larger and unevenly staining rods, which often show clubbing.

According to Clark and Ruehl (1919) who studied 70 strains of different species at brief intervals up to 48 hours and then at longer intervals for one week, striking changes in morphology take place during the early hours of growth. In all instances, except in the case of the diphtherial group, the organisms found in young cultures, 4 to 9 hours old, are larger than the forms found in 20- to 24-hour cultures. In the case of the diphtherial group, the young organisms, 2 to 6 hours old, are definitely smaller and more solid-staining than the older forms. Most of the strains do not attain the original size of the organisms inoculated until they are 12 to 18 hours old. These observations were, in part, confirmed by Henrici (1928) on a bacillus belonging to the diphtherial group. On the other hand, Albert (1921), working with 125 pure cultures of the diphtheria bacillus, did not corroborate the findings of Clark and Ruehl. He observed a slight increase in size during the first 4 hours, maintenance of that size

for about 20 hours, and then a rapid diminution after the 24-hour period. At the end of 7 days, the average length was only about one-third of the maximum. In regard to shape, the young bacilli, according to Albert, are rather bluntly pointed; with the development of granules, the ends become more rounded, even bulging. With the disappearance of the granules during the process of disintegration, the bacilli become irregular in shape with the ends again more pointed. Young bacilli stain solidly; but after a few hours, the protoplasm of some of the bacilli in certain areas takes a heavier stain than the intermediate portions, thus producing a barred appearance. The metachromatic granules appear in cultures 4 to 8 hours old. They are small at first, attaining their largest average size in cultures 12 to 15 hours old. After this period, the granules diminish in size and disappear. The percentage of bacteria containing granules increases rapidly from the 4-hour period, when there are but few granules, to the 12-hour period when 91 per cent of the bacteria contain granules. At the end of two days most of the bacilli have lost their granules.

Powell (1923) studied 299 single-cell cultures and found that they pass through different morphological phases similar to those derived directly from colonies. After a 4-hour incubation period on Loeffler's medium, the cultures contain only solid forms similar to the C^2 forms of Wesbrook. After 8 hours the morphology is still the same. After 12 hours the solid-staining forms still predominate but granular forms similar to the C forms begin to appear. After 24 hours all the cultures show a predominance of granular forms of the C type. A, B, and other granular forms are also present and some of the cultures show barred forms as well. During the next 24 hours the morphological picture remains about the same, except for the appearance of large irregular forms with many of the bacilli staining less distinctly with methylene blue. The single-cell cultures present the same morphological picture as do the corresponding colony cultures.

Customarily, observers view diphtherial cultures after 18 to 36 hours of incubation. In 1934, Solé introduced a method for

the rapid culturing of diphtheria bacilli; and since the reports of Brahdy *et al.* (1934, 1935) it is coming into greater vogue. For this method, sterile cotton swabs are impregnated with sterile, undiluted, unheated horse serum without preservative and heated over a flame to coagulate the surface. After swabbing the nose or throat, the swab is placed in a sterile, dry tube, incubated 2 to 4 hours and smears made directly from the swab. The diagnosis of such smears quite obviously necessitates a familiarity with the morphology of diphtheria bacilli in cultures 2 to 4 hours old, rather than the classical morphological pictures based on cultures 18 to 36 hours old.

Relation to pH of the culture medium. In acid media (+2 per cent), Denny (1903) observed that the organisms after 48 hours' incubation are short and sometimes oval in shape with well-marked granules. Alkaline media (−2 per cent) do not have as much effect on the morphology of the diphtheria bacilli as does an acid medium. Bunker (1917), on the other hand found that very acid media (pH not given) yield large, irregular, solid-staining forms in pure culture, while very alkaline media give minute, solid-staining, triangular forms, resembling Wesbrook's D^2 forms. Between these extremes practically all of Wesbrook's forms can be observed. Laybourn (1921) reported that acid media (about pH 6.1) give small, irregular forms with few granules, and that alkaline media induce minute, triangular forms with few or no granules. The observations of Morton (1935b) confirmed in part those of Denny and of Laybourn in that acid media (about pH 6.2) favor the production of small forms. However, a systematic examination of the effect of pH on the cell morphology of the diphtheria bacillus has yet to be made.

Presence of glucose in the culture medium. Heinemann (1917) and Yarisawa (1926) noticed that the presence of glucose favors the production of coccoid forms. This effect may perhaps be associated with the acidification of the medium as a result of the fermentation of the glucose; but acidification is not the entire explanation. Morton (1935b) confirmed these observations and in addition reported that media with a pH corresponding to that in tubes in which glucose is being fermented give small forms but these are not coccoid.

Relation to oxygen tension. Wherry (1917) observed that when a culture of the diphtheria bacillus is grown aerobically on Loeffler's medium the A^1, C^1, and D^1 types of Wesbrook predominate, whereas when the culture is grown anaerobically, the D^2 type predominates. In confirmation, Schneider (1931) and Beck (1933) likewise observed that anaerobic growth yields short, thick, solid-staining forms devoid of polar granules. In addition to studying the effect of oxygen on the morphology of *C. diphtheriae*, Beck studied the influence of other gases, as well. He found that H_2, CO_2 and H_2S, although without effect on the virulence of the microörganisms, do bring about changes in the morphology. The presence of hydrogen sulfide in the atmosphere results in a temporary increase in the rate of growth and in the formation of granules.

Morphology in various media. Coccoid forms of the diphtheria bacillus have been observed to arise in tryptic digest broth prepared from horse muscle (Parish, 1927); these forms reverted, however, to rod forms in the first subculture on Loeffler's medium. Parker (1928), employing tryptic serum agar, has noticed a somewhat similar phenomenon. Forty-seven per cent of 57 diphtherial strains are reported by Grubb and Koser (1934) as promptly changing their morphology to coccus forms when grown on liver infusion medium. Eighteen-hour cultures on inspissated horse serum are described as yielding short bacillary forms, whereas agar yields long forms (Barratt, 1924). The presence of blood in the medium is said to favor the production of granules (Megrail, 1922). Bile and bile salts are unique in that their presence in culture media is said to produce long filamentous forms with numerous branches and many metachromatic granules (Berthelot, Ramon, Grasset, and Amoureux, 1927; Grasset and Grasset, 1930). The presence of $CuSO_4$ in the culture medium has been observed to change the morphology of diphtheria bacilli to that of cocci (Pope and Pinfield, 1932; Morton 1935b, 1940). Morton has observed, in addition, that $MgSO_4$, Na_2SO_4, $Cu(NO_3)_2$, $MgCl_2$, and $(NH_4)_2SO_4$ have somewhat the same effect.

The addition of guinea pig blood to agar favors the formation of coccoid forms (Yarisawa, 1926). Sterile emulsions of guinea

pig liver or kidney when added to agar have been noticed by Gins and Jermoljewa (1929) to change the morphology of diphtheria bacilli to that of the Hofmann bacillus. This change has also been observed by Schneider (1931) and Malcherek (1932). The first subcultures to Loeffler's medium from the peritoneal fluid of a guinea pig, that had been injected 24 hours previously by Crowell (1926) with a single-cell culture of diphtheria bacilli, have yielded a pure culture of coccoid forms. Subsequent transplants to Loeffler's medium from the culture of coccus forms have yielded pure cultures of rod forms. Maver (1931) has confirmed Hadley's (1907) observations that coccoid and solid-staining forms are produced when diphtheria bacilli are cultured in proteid-free media. Maver emphasizes that when there is a sudden change in the composition of the culture medium, the organism shows marked variations in morphology but that upon continued cultivation on the same medium the morphology gradually reverts to the more common rod forms. These observations may be the explanation for the change in morphology noticed when diphtherial microörganisms are cultured directly from the animal body. McGuigan and Frobisher (1936) recommend that the diagnosis of diphtheria should not be based upon the microscopical examination of smears made directly from colonies on tellurite medium, because the organisms often are small, thick, coccoid, and solid-staining. Involution forms have been reported as being not so abundant on amniotic fluid agar as on Loeffler's medium (Giltner and Ludlum, 1916).

Temperature employed for sterilization of Loeffler's medium. The amount of heat employed during the sterilization of the Loeffler's medium influences the morphology of diphtherial microörganisms when cultured upon it (Yarisawa, 1926; Morton, 1935b). The medium heated at 80°C. gives best coccoid formation, but when heated at 90°C. it yields typical rod forms. Perry and Petran (1939) also recognize the importance of the temperature of sterilization of Loeffler's medium in this connection.

Morphology in a mixed culture with other organisms. Smirnow (1908) in studying some symbiotic relations of *C. diphtheriae*

with other microörganisms has observed that such relationships give rise to coccoid forms. The change in morphology is not permanent as the diphtheria bacilli return to their typical morphology when grown under favorable conditions. Stovall, Scheid and Nichols (1923) have found that when a pure culture of *C. diphtheriae* is mixed and grown with a pure culture of *Staphylococcus aureus*, the diphtheria bacilli change their morphology from large club-shaped organisms with large granules to small thin organisms staining with heavy bands. No such effect is observed when non-hemolytic streptococci are substituted for the *Staphylococcus aureus* culture.

Discussion. From the relatively few citations of the voluminous literature it is evident that many factors, in the diphtherial organism itself, and in its environment, produce marked effects on the morphology of the cells. These factors have been cited because of their immediate practical significance.

In the study of the colony forms produced by a bacterial species, much information relative to the colony phase of the species often can be obtained by studying the morphology of the microörganisms. In the case of the diphtherial microörganism, however, many unusual circumstances are present. The genus *Corynebacterium* is exceptional in that during the normal growth and development of the microörganisms, the individuals are smaller during the first few hours of growth, then gradually attain their normal size. In the case of microörganisms of other genera the individual cells are slightly larger during the first few hours of growth. The so-called "normal" cells for the diphtherial species may be (1) rod forms, either granular, granular-barred, barred, or solid-staining; or (2) filamentous and branching forms; or (3) spheroidal forms. The significance of these various cell forms is not clearly understood at the present time. All conditions in the environment being as uniform as possible, the microörganisms in the R colony form are characterized by long, filamentous forms which stain solidly and often show branching. Microörganisms in the SR colony forms show a greater tendency towards bizarre forms; while those in the S colony form are more uniform in outline. The

organisms in the D colony form are characterized by their small size.

The diphtherial species is also outstanding in that the cell morphology is readily and markedly changed by the environment. The physiological youth and age of the culture, the reaction of the medium, the gaseous environment, the composition of the medium, and the temperature employed for sterilizing the medium have a decided influence upon the cell morphology. The sudden change of the microörganisms from one medium to another often results in a pronounced variation in the cell morphology. This variation is usually temporary, however, since continued subculturing upon one kind of medium results in restoring the microörganisms to their usual morphology. While many of the common factors that cause a temporary alteration in the cell morphology are known, there are doubtless many others still to be discovered.

SUMMARY

The diphtherial species, which in the modern conception of a bacterial species implies not only the diphtheria "bacillus" but all the cultural and morphological elements, is like many others in that it displays a variety of colony forms, namely, the mucoid (M), smooth (S), intermediate (SR), rough (R), dwarf (D), and gonidial (G) colony forms. The mucoid colony form, rarely reported, has not been demonstrated as yet to be made up of capsulated organisms or of organisms containing large amounts of a type-specific substance. The SR colony form, intermediate between S and R, occupies a greater rôle in the life history of the diphtheral species than similar forms usually occupy in other bacterial species. It is characterized by a pellicle type of growth in liquid media; such growth insures an adequate supply of oxygen to the growing organisms and thus enables them to develop more rapidly, and more readily to produce toxin or to ferment various carbohydrates. The R form is likewise a bit unusual in the diphtherial species in that the R colonies are smaller than the S, whereas in most other species it is the other way around. These various colony forms may be stable over a fairly long

period of cultivation or they may undergo spontaneous or forced variation. The trend in variation is from S to SR to R and reversion to the SR, or occasionally to the S form. Dwarf (D) colony forms have been observed to arise from the S, SR, and R forms. One is not able to predict with certainty what forms will arise when a given colony form is subjected to forced dissociation. In all cases, cultures from the S, SR, R, and D colony forms can be readily identified as the diphtherial species by their reactions towards the various test carbohydrates and specific immune serum, their virulence for guinea pigs, and their elaboration of the specific toxin.

Correlated with these various colonial manifestations is the manner of growth in liquid media. The M and S forms grow as uniform, homogeneous suspensions, the R form as a precipitate on the bottom of the vessel, and the SR form as a slight turbidity at first, which is replaced by a pellicle and sediment with the broth becoming clear. The D form grows as a very light turbidity and fine sediment, whereas the G form often fails to impart visible signs of growth in filtrates for a considerable period of time. When visible growth does occur, it is of a very fine turbidity with a ropy sediment. Suspended in physiological saline, microörganisms from the S colonies produce a uniform turbidity, those from the R colonies clump spontaneously, whereas organisms from the SR colonies give indeterminate results. Another property that appears to be correlated with colony form is the hemolytic activity of the cells. The S forms are hemolytic, the R forms less so, and the D forms non-hemolytic. Diphtherial cultures show the greatest hemolytic activity when 48 hours old; the hemolysin is thermolabile and non-filterable. Other members of the genus *Corynebacterium* have not been observed to possess this hemolytic property.

While the diphtherial microörganism reduces potassium tellurite, the various colony forms show differences in the rate of reduction and the degree of pigmentation. The differences in the amount of pigmentation of the colonies are probably due to the shape of the colony, the moisture content, and the texture of the surface. The S colonies (called "mitis" by the English

workers) which are raised, moist, and shiny appear black and shiny on tellurite medium. The SR colonies (called "gravis" by the English workers) are flatter and dryer, and appear grayish and dry on tellurite medium. The D forms which are slow in bringing about biochemical reactions are also slower than the S and SR forms in becoming pigmented.

Diphtherial microörganisms may be either virulent or avirulent for guinea pigs. If a strain is virulent, organisms from all the colony forms (S, SR, R, and D) may be virulent. There are, however, differences in the number of organisms required to produce death of the guinea pig in a stated time, the SR form having been found to produce death with fewer organisms than the S form. Quantitative tests have not been made with the R and D forms. In man, organisms from the S ("mitis") colony form appear to produce in the fauces a greater superficial membrane which is firm in texture and tends to extend into the intrathoracic air passages; and the most frequent cause of death is the respiratory obstruction produced by membrane formation. Organisms from the SR ("gravis") colony form appear to produce in the fauces less superficial membrane which is less firm in texture and shows less tendency to extend into the intrathoracic air passages; but this type produced deeper penetration of the tissues of the inflamed parts and greater involvement of the cervical lymph glands and surrounding tissues. In this case, the most frequent cause of death is the toxic effect on the viscera, especially the heart and kidneys. Toxigenicity does not appear to be the complete explanation for virulence, since the most virulent strains are not necessarily the greatest or most rapid toxin producers. Diphtherial strains may be either toxigenic or non-toxigenic. If toxigenic, organisms in all the colony forms (S, SR, R and D) are capable of elaborating the toxin. There are, however, differences in the rate and amount of toxin produced. The important question of why certain diphtherial strains are virulent and toxigenic and other strains are not is still unanswered. These attributes of the diphtherial microörganism are not associated with any particular colony form, nor with a particular antigenic component or serological type, nor with any

known biochemical activity. Studies into the chemical composition and respiration of diphtheria bacilli have only recently begun, and should contribute valuable information on many of the obscure aspects of the physiology of the organism.

Another property of the diphtherial microörganism not associated with any particular colony form is serological specificity. Preliminary observations have shown that microörganisms in the S, SR, R, and D colony forms behave similarly towards type-specific sera. The antigen responsible for type specificity among diphtheria bacilli has not as yet been identified, but the chemical studies already under way by the Chinese investigators should furnish this much-needed information.

The fermentation reactions of the diphtherial microörganism do not vary qualitatively with the different colony forms, but there are variations in the rates and the amounts of acid produced. *C. diphtheriae* produces acid from glucose and usually not from sucrose, but there are exceptions. In view of the rarity with which sucrose is utilized and the uncertainties attending the older recorded instances of such utilization, a careful reinvestigation of this property by modern methods would be highly desirable. Acid is usually produced from dextrin, starch and glycogen, the latter two substances, especially, being more frequently attacked by organisms in the SR colony form than in the other colony forms, as judged by the usual fermentation tests. The first, and thus far the only, respiration studies of the different colony forms indicate that organisms in the S ("mitis") and SR ("gravis") colony forms alike utilize glycogen, whereas the usual fermentation tests show that only the SR forms utilize it, *i.e.*, produce acid. This sort of discrepancy emphasizes again the need for careful quantitative metabolic studies as a basis for reliable interpretation and characterization.

The cell morphology of the diphtherial microörganism is quite variable. In addition to the usual variations in morphological forms which accompany changes in colony form, extensive morphological changes take place during the normal growth of the microörganisms, changes that are greatly modified by the reaction of the medium, the composition and temperature of steriliza-

tion of the medium, oxygen tension and symbiotic relations with other microörganisms. In addition to showing a variety of sizes and shapes, the microörganisms may appear as granular, granular-barred, barred or solid staining rods which may show filaments or branching forms. They may also appear in spheroidal form which, in certain cases, is transitory and dependent upon the environment, and in others is more permanent and independent of the environment.

This review of the many isolated observations of earlier investigators and of the results of more recent studies on the diphtherial organism emphasizes the necessity for viewing the diphtherial species in a manner quite different from that heretofore demanded by the older monomorphic concept of a bacterial species. *C. diphtheriae* is not typified exclusively by the "bacillus" of Klebs and Loeffler but by a considerable diversity of morphological and cultural elements that, taken collectively and perhaps in a certain sequence, make up the diphtherial species.

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